EXHIBIT 1

Characterization and Enrichment of Cardiomyocytes Derived From Human Embryonic Stem Cells

Chunhui Xu, Shailaja Police, Namitha Rao, Melissa K. Carpenter

Abstract—Cell replacement therapy is a promising approach for the treatment of cardiac diseases, but is challenged by a limited supply of appropriate cells. We have investigated whether functional cardiomyocytes can be efficiently generated from human embryonic stem (hES) cells. Cardiomyocyte differentiation was evaluated using 3 parent (H1, H7, and H9) hES cell lines and 2 clonal (H9.1 and H9.2) hES cell lines. All cell lines examined differentiated into cardiomyocytes, even after long-term culture (50 passages or ≈260 population doublings). Upon differentiation, beating cells were observed after one week in differentiation conditions, increased in numbers with time, and could retain contractility for over 70 days. The beating cells expressed markers characteristic of cardiomyocytes, such as cardiac α-myosin heavy chain, cardiac troponin I and T, atrial natriuretic factor, and cardiac transcription factors GATA-4, Nkx2.5, and MEF-2. In addition, cardiomyocyte differentiation could be enhanced by treatment of cells with 5-aza-2′-deoxycytidine but not DMSO or retinoic acid. Furthermore, the differentiated cultures could be dissociated and enriched by Percoll density centrifugation to give a population containing 70% cardiomyocytes. The enriched population was proliferative and showed appropriate expression of cardiomyocyte markers. The extended replicative capacity of hES cells and the ability to differentiate and enrich for functional human cardiomyocytes warrant further development of these cells for clinical application in heart diseases. (*Circ Res.* 2002;91:501-508.)

Key Words: human embryonic stem cells ■ cardiomyocytes ■ differentiation ■ pharmacological responses ■ cell separation

Human cardiomyocytes proliferate and mature during gestation: however, these cells terminally differentiate soon after birth.[1] It is thus generally accepted that cardiomyocytes cannot be regenerated once heart tissue is damaged by trauma such as ischemic conditions leading to cardiac infarction.[1,2] Although it appears that somatic stem cells can migrate to heart tissue and differentiate into cardiomyocytes,[3,4] such events may not be sufficient to reverse the pathological conditions. To enhance the biological function of the damaged heart, cell transplantation may be an effective therapy. Animal studies have used various types of cells for transplantation, including fetal and neonatal cardiomyocytes, skeletal and smooth muscle, fibroblasts, and bone marrow–derived cells.[4-11] Many cell types including fetal and neonatal cardiomyocytes appear to be promising candidates because of their ability to integrate into the host tissue[7,12,13] and to improve heart function.[14,15] Although this type of transplantation is promising, the source of cells such as human fetal and neonatal cardiomyocytes for cell therapies is, however, limited. This issue is particularly relevant because a significant percentage of transplanted fetal rat cardiomyocytes die posttransplantation.[16] It may therefore require either transplantation of large numbers of cardiomyocytes to achieve survival of adequate cell numbers or improvement of survival of transplanted cells.

Cardiomyocytes have been successfully derived from mouse embryonic stem (mES) cells and shown to form stable grafts in the mouse heart.[17-23] The availability of human embryonic stem (hES) cells[24,25] offers a possible solution to the poor availability of human cardiomyocytes for transplantation. hES cells have been successfully maintained in vitro for over 250 population doublings and retain stable phenotype and karyotype.[26,27] Furthermore, we have established a feeder-free system for culturing hES cells that maintains the potential of these cells to differentiate into cells of all 3 germ lineages, including beating cardiomyocytes.[27] This culture system will facilitate generation of large quantities of cells for therapeutic applications.

In the present study, we report that cardiomyocytes can be efficiently derived from hES cells using appropriate culture conditions. The cells express cardiac genes and respond appropriately to cardioactive drugs. hES cell–derived cardiomyocytes can be enriched by density separation and appear to retain appropriate phenotype, which will facilitate their use in cell replacement therapy.

Materials and Methods

Induction of Cardiomyocyte Differentiation

hES cells were maintained as described in the expanded Materials and Methods section (which can be found in the online data

Original received March 28, 2002; revision received August 19, 2002; accepted August 19, 2002.
From the Geron Corporation, Menlo Park, Calif.
Correspondence to Chunhui Xu, PhD, Geron Corporation, 230 Constitution Dr, Menlo Park, CA 94025. E-mail cxu@geron.com

Circulation Research is available at http://www.circresaha.org

DOI: 10.1161/01.RES.0000035254.80718.91

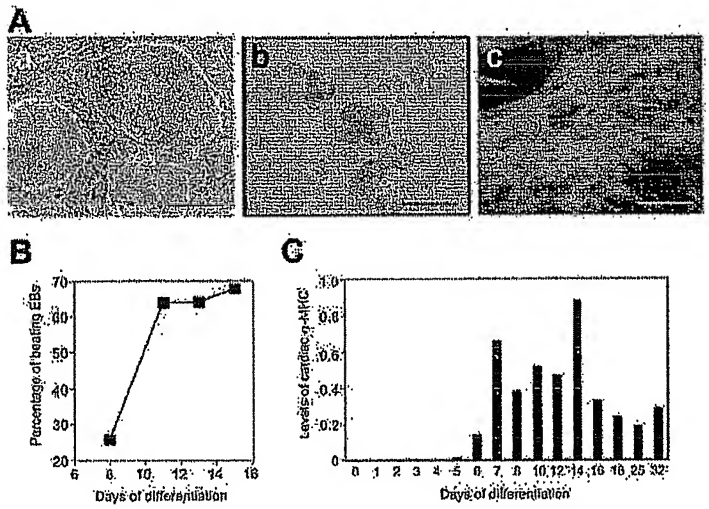

Figure 1. Differentiation of cardiomyocytes from hES cells. A, Confluent cultures of undifferentiated hES cells (a) were dissociated and cultured in suspension to form embryoid bodies (EBs) (b). EBs were transferred to gelatin-coated plates after 4 days in suspension culture to allow further differentiation into a heterogeneous cells, including spontaneously contracting cardiomyocytes that were positive for cTnI (c). Bar=400 μm for a and b and 50 μm for c. B, Percentage of EBs derived from H9.2 cells (passage 29+26, cells were subcloned from H9 at passage 29 and maintained for additional 26 passages) containing contracting cells during differentiation. C, Real-time RT-PCR analysis of cardiac α-MHC during differentiation of H1 cells (passage 29) normalized to 18S RNA.

supplement available at http://www.circresaha.org) and induced to differentiate as described below. Cells were dissociated into clumps using 200 U/mL collagenase IV (Invitrogen) at 37°C for 5 to 10 minutes and cultured in suspension using low attachment plates (Corning Inc) to form embryoid bodies (EBs). The differentiation medium contained 80% KO-DMEM, 1 mmol/L L-glutamine, 0.1 mmol/L β-mercaptoethanol, 1% nonessential amino acids stock, and 20% FBS (Hyclone). After 4 days in suspension, EBs were transferred onto gelatin or poly-L-lysine–coated plates at ≈1 to 3 EBs/cm^2 and cultured for additional days as described in Results. The cultures were then examined for the presence of beating cells and subjected to analysis of gene expression or pharmacological studies. The effect of the differentiation reagents dimethyl sulfoxide (DMSO), all-trans retinoic acid (RA), or 5-aza-2′-deoxycytidine (5-aza-dC), which are known to enhance cardiomyocyte differentiation in murine embryonic carcinoma (mEC) P19 cells, mES cells, or mesenchymal stem cells,[26–30] respectively, was assessed at different times during differentiation. Cultures were exposed to the reagent at the beginning of treatment and returned to basal medium without the reagent after the treatment. The number of days of differentiation includes the days in which the cells were maintained in suspension. For example, differentiation day 6 is after cells were maintained in suspension for 4 days, plated, and cultured for an additional 2 days after plating.

hES cell–derived cardiomyocytes were characterized by immunostaining and RT-PCR and evaluated in vitro for responses to pharmacological agents as described in the online data supplement.

Percoll Enrichment of Cardiomyocytes

Differentiated hES cells containing beating cells were dissociated, resuspended in differentiation medium, and loaded onto a discontinuous Percoll gradient. Percoll (Pharmacia) was diluted in a buffer containing 20 mmol/L HEPES and 150 mmol/L NaCl. The gradient consisted of a 40.5% Percoll layer over a layer of 58.5% Percoll. After centrifugation at 1500*g* for 30 minutes, cell layers were apparent. Cells at different layers were collected, washed, resuspended in the differentiation medium, and plated for immunostaining, or collected for real-time RT-PCR analysis. For immunocytochemical analysis, the fractionated cells were seeded into chamber slides, cultured for an additional few days and immunostained.

Methods for dissociation of cardiomyocytes, immunostaining and RT-PCR are provided in the online data supplement.

Results

Cardiac differentiation was initiated by inducing EB formation from undifferentiated hES cells (Figure 1A). In order to monitor the presence of beating cells in individual EBs, EBs were seeded at low density after 4 days in suspension culture, and the locations of EBs in each well were recorded. The EBs attached and continued to proliferate and differentiate into a heterogeneous population of cells including beating cardiomyocytes. Spontaneously contracting cells appeared as clusters and were identified in approximately 25% of the individual EBs at differentiation day 8 and increased to as many as 70% of the EBs by day 16 (Figure 1B). The percentage of beating EBs usually increased over time until day ≈20 and maintained at this level. In some cases, the number of beating EBs declined due to the overgrowth of other cells, which sometimes caused the peeling of cells from the plate. We found that this problem can be overcome by lowering the EB seeding density, more gently aspirating during medium exchanges, or dissociating the cells and then replating them. In our hands, contracting cells could be found in long-term cultures maintained up to differentiation day 70.

Cardiomyocyte formation in EB cultures was seen in 3 hES cell lines as well as 2 clonal lines tested (H1, H7, H9, H9.1, and H9.2). hES cells maintained for 50 passages (≈260 population doublings) retained the capacity to differentiate into cardiomyocytes (see an example in Figure 1B).

Expression of Cardiac Markers in hES-Derived Cardiomyocytes

hES cell–derived cardiomyocytes express cardiac-specific troponin I (cTnI), a subunit of the troponin complex that provides a calcium-sensitive molecular switch for the regulation of striated muscle contraction.[31] We found that cTnI was detected only in the beating regions of the culture. A representative cTnI-positive area is shown in Figure 1Ac. The presence of cTnI in the contracting cells was also confirmed by Western blot, which showed that cTnI was expressed in differentiated hES cultures containing contracting cells, but not in undifferentiated hES cells or differentiated cultures with no evidence of contracting cells (data not shown). Similar results were found in all cell lines tested.

Real-time RT-PCR assays showed that cardiac-specific α-MHC transcripts were undetectable in undifferentiated hES cell cultures or differentiated cultures at early stages, and

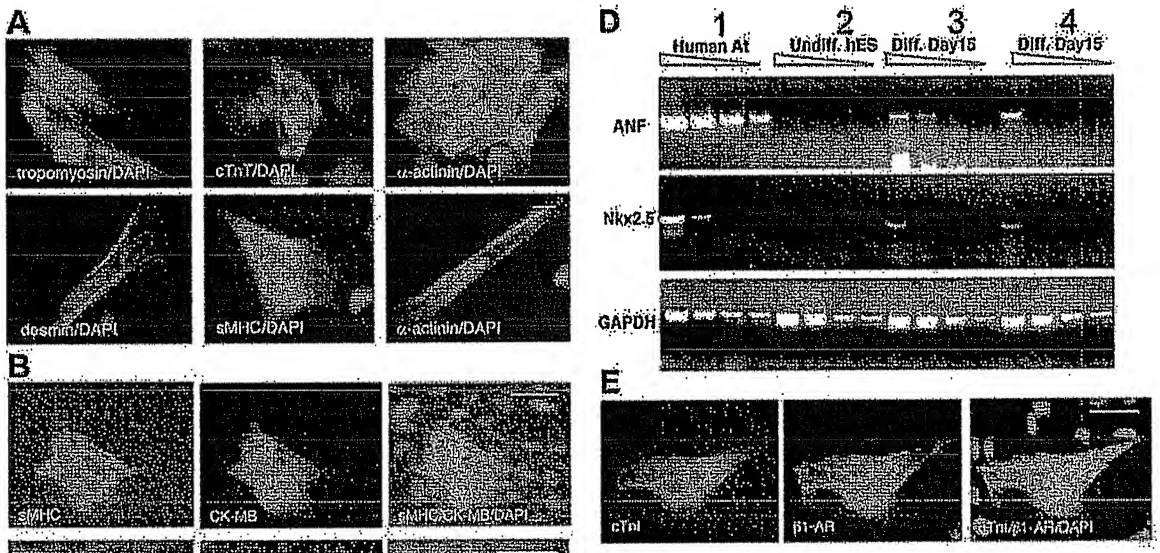

Figure 2. Marker analysis of hES cell–derived cardiomyocytes. A, H7 cells (passage 37), dissociated at differentiation day 29, cultured for an additional 3 days and assessed for tropomyosin immunoreactivity. H9 cells (passage 28) were dissociated at differentiation day 15, cultured for an additional 2 days and stained with sMHC, α-actinin, desmin, and cTnT as indicated. Bar=10 μm. B, H9 cells (passage 24) at differentiation day 11 were partially dissociated, further cultured for 10 days and stained with antibodies against sMHC and CK-MB or cTnI and myoglobin. C, H9 cells (passage 23) at differentiation day 14 were partially dissociated, further cultured for 10 days and stained with antibodies against GATA-4 or MEF-2 and cTnI as indicated. D, RT-PCR analysis of Nkx2.5, ANF, and GAPDH in H9 undifferentiated cells (passage 24, sample 2), two independent differentiated cultures at differentiation day 15 (sample 3 and 4) and human fetal atrial cDNA (sample 1). Samples of cDNA and three 10-fold serial dilutions were analyzed to compare the levels of mRNA. E, Cells derived from H1 cells (passage 30) at differentiation day 22 were stained with antibodies against cTnI and β1-AR. DAPI was used for staining nuclei for A, B, C, and E. Bar=50 μm for B, C, and E.

increased significantly after day 7 of differentiation (Figure 1C). In contrast, expression of hTERT, a gene expressed in undifferentiated hES cell cultures,[27,32] decreased during the process of differentiation (data not shown).

Other muscle markers were evaluated using dissociated hES cell–derived cardiomyocytes: sMHC, tropomyosin, α-actinin, desmin, and cardiac troponin T (cTnT) proteins were detected in single beating cells or clusters of cells (Figure 2A). Single stained cardiomyocytes showed spindle, round, and tri- or multiangular morphologies with striations characteristic of the sarcomeric structures of muscle cells. Immunostaining showed that 100% of sMHC-positive cells express cTnI, indicating that all the identified cells were cardiomyocytes. Furthermore, myogenin, a skeletal muscle–specific marker, was not detectable in the sMHC-positive cells by immunostaining, suggesting that the hES cell–derived cardiomyocytes were not expressing inappropriate proteins.

In addition to structural proteins, creatine kinase-MB (CK-MB) and myoglobin were also expressed by hES cell–derived cardiomyocytes (Figure 2B). CK-MB is found to be involved in high-energy phosphate transfer and facilitates diffusion of high-energy phosphate from mitochondria to myofibril in myocytes. Myoglobin is a cytosolic oxygen binding protein responsible for the storage and diffusion of oxygen within myocytes. Thus, hES cell–derived cardiomyocytes appear to have appropriate metabolic activity.

hES cell–derived cardiomyocytes also specifically expressed several cardiac transcription factors, including GATA-4, MEF-2, and Nkx2.5, in the differentiated cultures. These transcription factors are expressed in precardiac mesoderm and persist in the heart during development. GATA-4 immunoreactivity was found in nuclei of all cTnI-positive cells (Figure 2C). Western blots also indicated that GATA-4 is highly expressed in differentiated hES cells containing contracting cells but not in differentiated cultures that did not contain contracting cells (data not shown), indicating that GATA-4 is associated with cardiomyocyte differentiation. Similarly, MEF-2 was also expressed in nuclei of cTnI-positive cells as detected by immunostaining (Figure 2C).

Semiquantitative RT-PCR indicated that Nkx2.5 was expressed in hES cell–differentiated cultures containing beating cardiomyocytes, but undetectable in undifferentiated cultures (Figure 2D). Real-time RT-PCR analysis indicated that expression of Nkx2.5 is very low or nondetectable during H1 differentiation from day 0 to 6 and significantly increased at day 7 (data not shown). Therefore, hES cell–derived cardiomyocytes express cardiac transcription factors appropriately.

In addition, atrial natriuretic factor (ANF), a hormone that is actively expressed in both atrial and ventricular cardiomyocytes in developing heart, but is significantly downregulated in adult ventricular cells,[33] was found to be up-regulated during cardiac differentiation of hES cells as detected by a semiquantitative RT-PCR (Figure 2D).

Taken together, the above data indicate that hES cell–derived cardiomyocytes show characteristic gene expression patterns of developing cardiomyocytes.

Pharmacological Responses of hES Cell–Derived Cardiomyocytes

The in vitro function of hES cell–derived cardiomyocytes was examined by evaluating chronotropic effects of cardioactive drugs. Ion channels including L-type calcium channels play critical roles in cardiac contractile function.[34] RT-PCR analysis shows that α_1 subunit of L-type calcium channel is detected in differentiated cultures (data not shown). Therefore, we determined the effect of diltiazem, an ion channel blocker, on the beating frequency of hES cell–derived cardiomyocytes. Differentiated cells were incubated with various concentrations of the drug followed by measuring the beating frequency. Figure 3A shows that the beating frequency was decreased by diltiazem in a concentration-dependent manner; treatment with 10^{-7} mol/L diltiazem significantly reduced the frequency, and treatment with 10^{-5} mol/L stopped pulsatile contraction entirely. Contractions recovered to a normal rate 24 to 48 hours after removal of the drug. These results suggest functional ion channels exist in the hES cell–derived beating cardiomyocytes.

Cytosolic calcium is a crucial factor for controlling cardiomyocyte contraction and can be influenced by the interaction of adrenoceptors (ARs) with their ligands.[35] We therefore examined whether hES cell–derived cardiomyocytes expressed ARs by immunostaining with antibodies against AR and cTnI. The cardiomyocytes identified by cTnI expression were also immunoreactive for β_1-AR (Figure 2E) and α_1-AR (data not shown). To determine if ARs were functioning appropriately, contracting cells were treated with isoprenaline, a β_1-AR agonist, or phenylephrine, an α_1-AR agonist, and the rate of beating was monitored. As shown in Figures 3B and 3C, both isoprenaline and phenylephrine enhanced the contraction rate of hES cell–derived cardiomyocytes at differentiation day 15 to 20 in a dose-dependent manner. Unlike responses to isoprenaline or phenylephrine, cells at early stages (differentiation day 22 and 39) did not respond to clenbuterol, a β_2-AR agonist. However, cultures allowed to differentiate for a longer period of time (day 61 to 72) showed an increase in beating frequency (Figure 3E). These results suggest that differential responses of adrenoceptors occur

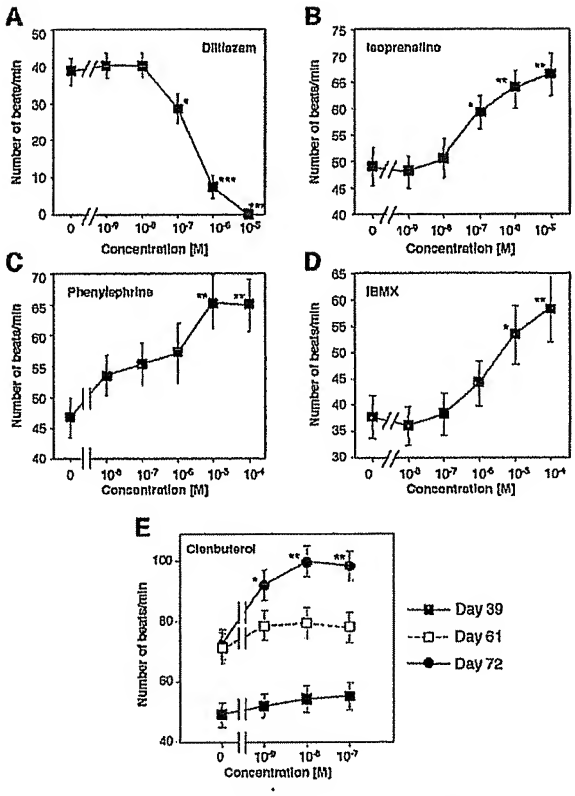

Figure 3. Studies of pharmacological responses. Effect of (A) diltiazem (a calcium channel blocker), (B) isoprenaline (a β_1-adrenoceptor agonist), (C) phenylephrine (an α1-adrenoceptor agonist), and (D) IBMX (an inhibitor of phosphodiesterases), on the contraction rate of cardiomyocytes derived from H9 cells (passage 31 to 32) or H7 cells (passage 49) at differentiation day 15 to 21. Effect of (E) clenbuterol (a β_2-adrenoceptor agonist) on H7 cells (passage 48) at differentiation day 39, 61, or 72. Each data point represents the mean±SEM pulsation rate. Statistical significance was tested by the ANOVA test: *P<0.05, **P<0.005, ***P<0.0005.

during cardiomyocyte differentiation from hES cells, similar to that seen with mES cell–derived cardiomyocytes.[36]

Application of isobutyl methylxanthine (IBMX), an inhibitor of phosphodiesterase (which converts cAMP into 5′AMP), resulted in a concentration-dependent increase of the contraction rate by IBMX (Figure 3D). These results indicate that the hES cell–derived cardiomyocytes respond appropriately to cardioactive drugs and this response may be mediated through a cAMP-dependent mechanism.[37]

Effect of Differentiation Induction Reagents on Cardiomyocyte Differentiation

In order to enhance cardiomyocyte differentiation, the effect of differentiation induction reagents was evaluated. DMSO and RA, which have been shown to induce cardiomyocyte differentiation in mEC P19 cells[28] and mES cells,[29] respectively, were evaluated but did not enhance hES cell cardiomyocyte differentiation (additional results in the online data supplement).

5-aza-dC has been shown to induce differentiation of mesenchymal stem cells presumably via demethylation of

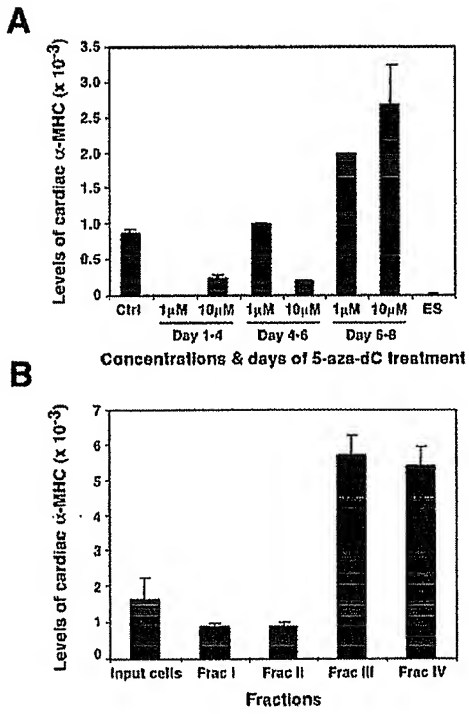

Figure 4. Enrichment of cardiomyocytes by 5-aza-dC treatment and Percoll separation. A, Effect of 5-aza-dC treatment on cardiac α-MHC mRNA levels of differentiation of H9 cells (passage 26). Cells were treated with 5-aza-dC at differentiation day 1 to 4, 4 to 6, or 6 to 8 and analyzed at differentiation day 15 for cardiac α-MHC mRNA levels by real-time RT-PCR Taqman analysis. Error bars that are not visible are smaller than the width of the symbol. ES indicates undifferentiated hES cells; Ctrl, untreated differentiated cell control. B, Effect of Percoll separation on enrichment of cardiomyocytes. H9 cells (passage 31) at differentiation day 22 were dissociated and separated by Percoll centrifugation. Cardiac α-MHC mRNA levels of cells in different fractions were compared with the starting material (input cells). 18S was used for normalization for A and B.

DNA.[30] To examine if 5-aza-dC affects cardiomyocyte differentiation of hES cells, hES cells were treated with 5-aza-dC at differentiation day 1 to 4, 4 to 6, or 6 to 8. Cells were harvested at day 15 and analyzed for cardiac α-MHC by real-time RT-PCR. Treatment of H9 or H1 cells with 5-aza-dC at day 6 to 8 significantly increased the expression of cardiac α-MHC (H9; data shown in Figure 4A). In contrast, a significant decrease in expression of cardiac α-MHC was observed when H9 or H1 cells were treated at differentiation day 1 to 4. In addition, the level of cardiac α-MHC decreased when H9 cells were treated with 10 μmol/L but not 1 μmol/L 5-aza-dC during differentiation day 4 to 6 compared with the nontreatment control. Immunostaining analysis of cTnI-positive cells indicated that the increase in α-MHC correlates with an increase in the number of cardiomyocytes (online data supplement). Therefore, 5-aza-dC appears to enhance cardiomyocyte differentiation from hES cells in a time-dependent manner. Further research is needed to characterize the complete phenotype of these cells.

Enrichment of Cardiomyocytes Using Discontinuous Percoll Gradients

In order to use hES cell–derived cardiomyocytes in therapeutic applications, it will be beneficial to produce a population of cells highly enriched for cardiomyocytes. We have used discontinuous Percoll gradients to successfully enrich hES cell–derived cardiomyocytes. An example is provided in online Table 2 (in the online data supplement available at http://www.circresaha.org) in which H7 cell–derived cardiomyocytes at differentiation day 21 were dissociated and applied to a discontinuous Percoll gradient (40.5% over 58.5%). After centrifugation, 2 layers of cells were observed: one on top of the Percoll (fraction I) and a layer of cells at the interface of the 2 layers of Percoll (fraction III). These 2 fractions, cells within the 40.5% Percoll layer (fraction II) and the 58.5% Percoll layer (fraction IV), and the starting material (input cells) were collected and cultured for 2 or 7 days before immunostaining. Although beating cells were observed in all fractions, fraction III and IV contained a higher percentage of beating cells. Quantitative analysis of triplicate wells showed that fraction III contained 36±3% sMHC-positive cells and fraction IV contained 70±5% sMHC-positive cells, whereas fraction I or II contained only 3% to 5% sMHC-positive cells 2 days after seeding (online Table 2). Compared with the starting material that contained 17±4% sMHC-positive cells, fraction IV showed a 4-fold enrichment. Similar results were observed for cells cultured for additional 7 days (online Table 2). We also applied the same separation procedure to H9 cells at differentiation day 22 and found that levels of cardiac α-MHC RNA in fractions III and IV were significantly higher than cells without the separation, confirming the enrichment (Figure 4B). Similar enrichment results (20% to 40% sMHC or cTnI-positive cells for fraction III and 50% to 70% sMHC or cTnI-positive cells for fraction IV) were observed in multiple experiments using H1 or H7 cells. These results indicate a significant enrichment of cardiomyocytes using a discontinuous Percoll gradient separation.

To characterize the Percoll-enriched cell populations, we performed immunostaining using antibodies against various markers. As shown in online Table 3, positive immunoreactivity for antibodies against cardiac α/βMHC, βMHC and sMHC was found in all cardiac cells as identified by cTnI-positive cells, but not in noncardiac cells. A representative image of cTnI and sMHC staining is shown in Figure 5. In addition, cTnI-positive cells expressed N-cadherin. Neither cardiac cells nor noncardiac cells expressed myogenin, AFP, or β-tubulin III, indicating the absence of skeletal muscle, endoderm cell types, or neurons in the Percoll-enriched culture. To examine if there were any undifferentiated hES cells in the population, surface markers for undifferentiated hES cells, SSEA-4 and Tra1-81, were analyzed. No detectable signal was found in either cardiac or noncardiac cells. Therefore, the Percoll-enriched cells did not appear to contain undifferentiated hES cells.

It has been reported that α-smooth muscle actin (SMA) is present in embryonic and fetal but not in adult cardiomyocytes.[38,39] Immunostaining results indicated that all cTnI-positive cells and a subset of cTnI-negative cells expressed

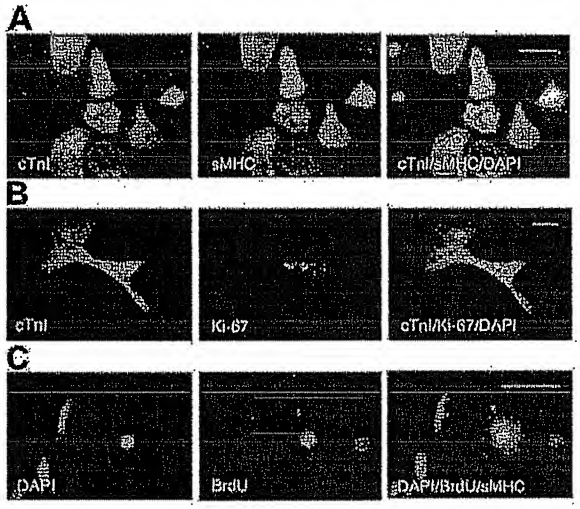

Figure 5. Characterization of Percoll-enriched cells. A, Immunostaining of H1 cells (passage 30) isolated at differentiation day 19, cultured for 2 days, and stained with antibodies against cTnI and sMHC. B, Immunostaining of H7 cells (passage 47) isolated at differentiation day 12, cultured for an additional 10 days, and stained with antibodies against cTnI and Ki-67. C, H7 cells (passage 37) isolated at differentiation day 29, cultured for additional 3 days, pulsed with BrdU, and stained with antibodies against sMHC and BrdU. Bar=50 μm

SMA, suggesting that these cardiomyocytes may represent an early stage of cardiomyocytes.

To evaluate the proliferative capacity of these cells, cultures were analyzed for BrdU incorporation and Ki-67 expression. Ki-67 is a protein in active phases of the cell cycle (G1, S, G2, and mitosis) but not in resting G0 cells and therefore used to assess cell proliferation.[40,41] In this experiment, H7 cells (passage 37) at differentiation day 13 were dissociated and isolated by Percoll separation. Cells in fraction III and IV were replated, cultured for additional 2 days, and then pulse-labeled with BrdU for 24 hours. We found that 43±4% of the sMHC-positive cells expressed BrdU, indicating that these cardiac cells were in S phase of proliferation. Parallel cultures were Percoll-separated at differentiation day 29, cultured for additional 4 days, and assessed for BrdU incorporation and the presence of Ki-67. We found that 23±10% of sMHC-positive cells incorporated BrdU and 28±4% of sMHC-positive cells were positive for Ki-67. In sMHC-negative cells, 71±2% cells incorporated BrdU and 46±7% cells were positive for Ki67. Experiments using other cultures also indicated that a subset of cTnI-positive cells expressed Ki-67 (online Table 3). Figure 5 shows a representative image. These results indicate that some of the hES cell–derived cardiomyocytes were proliferating.

Discussion

The generation of functional cardiomyocytes from hES cells has several potential applications including myocardial repair through cell transplantation. Such an application has already been demonstrated in animal models using other sources of cells[4–11]; however, the plasticity of adult stem cells has been recently challenged.[42,43] The assumed capacity of transdifferentiation of the adult stem cells into other lineages in vivo might simply be a fusing with existing cell types rather than direct conversion. In addition, adult stem cells usually have limited proliferative capacity, whereas hES cells have extended replicative capacity.[26] Therefore, hES cell–derived cardiomyocytes may prove to be the best candidate population for cell therapy. This and other potential applications of hES cell–derived cardiomyocytes are, however, largely dependent on practical aspects of producing a sufficient amount of these cells.

Our data demonstrate that hES cells can effectively differentiate into functional cardiomyocytes. This conclusion is based on (1) the contractility of the differentiated cultures, (2) specific expression of multiple cardiac-associated molecular markers by the differentiated cells, and (3) appropriate response of these differentiated cells to cardioactive drugs. While this article was in preparation, Kehat et al[44] reported that cardiomyocytes can be produced from H9.2 hES cells. In the present study, we report that cardiomyocytes can be generated from multiple hES cell lines tested (H1, H7, H9, H9.1, and H9.2) and that, using the H9.2 cells, we observed a higher percentage of beating EBs (70% versus 8%) compared with the earlier report. The difference in the efficiency of cardiomyocyte differentiation may reflect differences in culture conditions of the undifferentiated hES cells, methods used for the dissociation of hES cells to generate EBs, the length of EB suspension culture, and/or the quality of serum used for differentiation. For example, we have been maintaining undifferentiated hES cells on MEF feeders or in feeder-free conditions using medium containing serum replacement. However, Kehat et al cultured cells on feeders in medium containing FBS. Different culture conditions could lead to a different status of the hES cells used for differentiation and may be influenced by the confluence of the culture and amount of undifferentiated versus spontaneously differentiated cells in the cell population. In our experiments, cells were harvested using 200 U/mL collagenase IV for 5 to 10 minutes and gently dissociated into cell clumps for EB formation. These clumps vary in size, but the majority contained ≈100 cells or more. However, Kehat et al treated cells with 1 mg/mL collagenase IV for 20 minutes, which resulted in smaller clumps containing 3 to 20 cells. In addition, we allowed the EBs to attach onto plates after culture in suspension for only 4 days instead of 10 days as described by Kehat et al.[44] It is likely that the microenvironment within the EB culture will influence the differentiation of the cell population.

We have found that cardiomyocyte differentiation can be significantly enhanced by treatment of cells with 5-aza-dC, a demethylation reagent. This might reflect a direct improvement of cardiomyocyte differentiation due to regulation of gene expression by demethylation. Alternatively, it might simply be a net effect from the lowered efficiency of hES cell differentiation into other cell types. Our observation underscores the importance of demethylation for hES cell differentiation into cardiomyocytes and perhaps other cell types as well.

We and others have previously reported that hES cells have different properties than mES cells, including surface marker expression and response to growth factors.[24–27] Consistent with this observation, hES cell cardiomyocyte differentiation is indeed quite different from cardiomyocyte differentiation from mES and mEC cells. We observed cardiomyocyte differentiation from hES cells maintained for 260 population doublings, although cardiomyocyte differentiation using late passages of mES cells has been difficult. Whereas DMSO and RA enhance mEC P19 or mES cell cardiogenesis,[28,29] these compounds did not show such an effect on hES cell cardiomyocyte differentiation. Although the exact mechanism is unclear, it is possible that cardiomyocyte differentiation from hES cells is controlled by different signaling pathways or a common pathway that is also regulated by species-specific modulators. The effects of RA we have observed are in contrast to those reported by Schuldiner et al,[15] who showed that RA treatment increased expression of cardiac α-actin in H9.1 clonal cell line. This difference may have resulted from several factors such as different cell lines or subclones, culture systems, differentiation protocols, and/or the assay endpoints used.

In addition, we have also demonstrated the enrichment of hES cell–derived cardiomyocytes by Percoll gradient separation and proliferation capacity of the enriched cells. These cells express appropriate cardiomyocyte-associated proteins. A subset of them appears to be proliferative as determined by BrdU incorporation or expression of Ki-67, suggesting that these cardiomyocytes represent an early stage of cells. This population may be a useful model for studying cell cycle regulation of the cardiomyocytes. It will be important to determine if this represents an expandable population of cells.

In summary, we have demonstrated that an enriched population of cardiomyocytes can be derived from hES cells. These hES cell–derived cardiomyocytes can now be tested for their ability to enhance cardiac function in preclinical animal models and for utility in drug discovery.

Acknowledgments

This research is supported by the Geron Corporation. We thank Kathy Golds and Xiaoming Hao for skillful technical assistance, Dr Nam Kim for developing real-time RT-PCR analysis of cardiac α-MHC, and Drs Joseph Gold, Choy-Pik Chiu, Jane Lebkowski, Calvin Harley, Michael Schiff, and David Earp for insightful discussions and critical review of the manuscript.

References

1. MacLellan WR, Schneider MD. Genetic dissection of cardiac growth control pathways. *Annu Rev Physiol*. 2000;62:289–319.
2. Soonpaa MH, Field LJ. Survey of studies examining mammalian cardiomyocyte DNA synthesis. *Circ Res*. 1998;83:15–26.
3. Quaini F, Urbanek K, Beltrami AP, Finato N, Beltrami CA, Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Chimerism of the transplanted heart. *N Engl J Med*. 2002;346:5–15.
4. Jackson KA, Majka SM, Wang H, Pocius J, Hartley CJ, Majesky MW, Entman ML, Michael LH, Hirschi KK, Goodell MA. Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J Clin Invest*. 2001;107:1395–1402.
5. Li RK, Weisel RD, Mickle DA, Jia ZQ, Kim EJ, Sakai T, Tomita S, Schwartz L, Iwanochko M, Husain M, Cusimano RJ, Burns RJ, Yau TM. Autologous porcine heart cell transplantation improved heart function after a myocardial infarction. *J Thorac Cardiovasc Surg*. 2000;119:62–68.
6. Li RK, Yau TM, Weisel RD, Mickle DA, Sakai T, Choi A, Jia ZQ. Construction of a bioengineered cardiac graft. *J Thorac Cardiovasc Surg*. 2000;119:368–375.
7. Soonpaa MH, Koh GY, Klug MG, Field LJ. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. *Science*. 1994;264:98–101.
8. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med*. 2001;7:430–436.
9. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A, Anversa P. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701–705.
10. Kessler PD, Byrne BJ. Myoblast cell grafting into heart muscle: cellular biology and potential applications. *Annu Rev Physiol*. 1999;61:219–242.
11. Taylor DA, Atkins BZ, Hungspreugs P, Jones TR, Reedy MC, Hutcheson KA, Glower DD, Kraus WE. Regenerating functional myocardium: improved performance after skeletal myoblast transplantation. *Nat Med*. 1998;4:929–933.
12. Reinecke H, Zhang M, Bartosek T, Murry CE. Survival, integration, and differentiation of cardiomyocyte grafts: a study in normal and injured rat hearts. *Circulation*. 1999;100:193–202.
13. Koh GY, Soonpaa MH, Klug MG, Pride HP, Cooper BJ, Zipes DP, Field LJ. Stable fetal cardiomyocyte grafts in the hearts of dystrophic mice and dogs. *J Clin Invest*. 1995;96:2034–2042.
14. Sakai T, Li RK, Weisel RD, Mickle DA, E. J. K, Tomita S, Jia ZQ, Yau TM. Autologous heart cell transplantation improves cardiac function after myocardial injury. *Ann Thorac Surg*. 1999;68:2074–2080.
15. Li RK, Jia ZQ, Weisel RD, Mickle DA, Zhang J, Mohabeer MK, Rao V, Ivanov J. Cardiomyocyte transplantation improves heart function. *Ann Thorac Surg*. 1996;62:654–660; discussion, 660–661.
16. Zhang M, Methot D, Poppa V, Fujio Y, Walsh K, Murry CE. Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. *J Mol Cell Cardiol*. 2001;33:907–921.
17. Maltsev VA, Rohwedel J, Hescheler J, Wobus AM. Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. *Mech Dev*. 1993;44:41–50.
18. Maltsev VA, Wobus AM, Rohwedel J, Bader M, Hescheler J. Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ Res*. 1994;75:233–244.
19. Maltsev VA, Ji GJ, Wobus AM, Fleischmann BK, Hescheler J. Establishment of β-adrenergic modulation of L-type Ca^{2+} current in the early stages of cardiomyocyte development. *Circ Res*. 1999;84:136–145.
20. Hescheler J, Fleischmann BK, Lentini S, Maltsev VA, Rohwedel J, Wobus AM, Addicks K. Embryonic stem cells: a model to study structural and functional properties in cardiomyogenesis. *Cardiovasc Res*. 1997;36:149–162.
21. Miller-Hance WC, LaCorbiere M, Fuller SJ, Evans SM, Lyons G, Schmidt C, Robbins J, Chien KR. In vitro chamber specification during embryonic stem cell cardiogenesis: expression of the ventricular myosin light chain-2 gene is independent of heart tube formation. *J Biol Chem*. 1993;268:25244–25252.
22. Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J Clin Invest*. 1996;98:216–224.
23. Wobus AM, Rohwedel J, Maltsev V, Hescheler J. Development of cardiomyocytes expressing cardiac-specific genes, action potentials, and ionic channels during embryonic stem cell-derived cardiogenesis. *Ann N Y Acad Sci*. 1995;752:460–469.
24. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998;282:1145–1147.
25. Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. *Nat Biotechnol*. 2000;18:399–404.
26. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol*. 2000;227:271–278.

27. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotech.* 2001;19:971–974.
28. Edwards MK, Harris JF, McBurney MW. Induced muscle differentiation in an embryonal carcinoma cell line. *Mol Cell Biol.* 1983;3:2280–2286.
29. Wobus AM, Kaomei G, Shan J, Wellner MC, Rohwedel J, Ji G, Fleischmann B, Katus HA, Hescheler J, Franz WM. Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. *J Mol Cell Cardiol.* 1997; 29:1525–1539.
30. Fukuda K. Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering. *Artif Organs.* 2001;25:187–193.
31. Bhavsar PK, Brand NJ, Yacoub MH, Barton PJ. Isolation and characterization of the human cardiac troponin I gene (TNNI3). *Genomics.* 1996; 35:11–23.
32. Lebkowski JS, Gold J, Xu C, Funk W, Chiu CP, Carpenter MK. Human embryonic stem cells: culture, differentiation, and genetic modification for regenerative medicine applications. *Cancer J.* 2001;7(suppl 2):S83–S93.
33. Zeller R, Bloch KD, Williams BS, Arceci RJ, Seidman CE. Localized expression of the atrial natriuretic factor gene during cardiac embryogenesis. *Genes Dev.* 1987;1:693–698.
34. Trautwein W, Hescheler J. Regulation of cardiac L-type calcium current by phosphorylation and G proteins. *Annu Rev Physiol.* 1990;52:257–274.
35. Frey N, McKinsey TA, Olson EN. Decoding calcium signals involved in cardiac growth and function. *Nature Medicine.* 2000;6:1221–1227.
36. Wobus AM, Wallukat G, Hescheler J. Pluripotent mouse embryonic stem cells are able to differentiate into cardiomyocytes expressing chronotropic responses to adrenergic and cholinergic agents and Ca^{2+} channel blockers. *Differentiation.* 1991;48:173–182.
37. Post SR, Hammond HK, Insel PA. β-Adrenergic receptors and receptor signaling in heart failure. *Annu Rev Pharmacol Toxicol.* 1999;39: 343–360.
38. Leor J, Patterson M, Quinones MJ, Kedes LH, Kloner RA. Transplantation of fetal myocardial tissue into the infarcted myocardium of rat: a potential method for repair of infarcted myocardium? *Circulation.* 1996; 94:II332–II336.
39. Etzion S, Battler A, Barbash IM, Cagnano E, Zarin P, Granot Y, Kedes LH, Kloner RA, Leor J. Influence of embryonic cardiomyocyte transplantation on the progression of heart failure in a rat model of extensive myocardial infarction. *J Mol Cell Cardiol.* 2001;33:1321–1330.
40. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. *J Cell Physiol.* 2000;182:311–322.
41. Gilbus NE, Jones M, Turley H, Gatter KC, Nagvekar N, Newsom-Davis J, Willcox N. Oncogene proteins and proliferation antigens in thymomas: increased expression of epidermal growth factor receptor and Ki67 antigen. *J Clin Pathol.* 1995;48:447–455.
42. Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. *Nature.* 2002; 416:542–545.
43. Ying QL, Nichols J, Evans EP, Smith AG. Changing potency by spontaneous fusion. *Nature.* 2002;416:545–548.
44. Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, Livne E, Binah O, Itskovitz-Eldor J, Gepstein L. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *J Clin Invest.* 2001;108:407–414.
45. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. From the cover: effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A.* 2000;97:11307–11312.

EXHIBIT 2

Online Data Supplement (Xu et al.)

Materials and Methods

hES cell culture

hES cell lines, H1, H7, H9, H9.1 and H9.2,[1,2] were initially maintained on feeders in ES medium containing 80% knockout Dulbecco's modified Eagle's medium (KO-DMEM) (Invitrogen, Carlsbad, CA), 1 mM L-glutamine, 0.1 mM β-mercaptoethanol,1% nonessential amino acids stock (Invitrogen), 20% Serum Replacement (Invitrogen) and 4 ng/ml hbFGF (Invitrogen)[2] and were later maintained using feeder-free conditions as described previously.[3] Briefly, feeder-free cultures were passaged by incubation in 200 units/ml collagenase IV for 5-10 minutes at 37℃, dissociated and then seeded onto Matrigel®-coated plates and maintained in conditioned medium prepared from primary mouse embryonic fibroblast cultures. The mouse embryonic fibroblasts were derived from CF-1 E13 embryos as described.[4]

Dissociation of cardiomyocytes

Differentiated cultures containing beating cardiomyocytes were washed with PBS or a low-calcium solution containing 120 mM NaCl, 5.4 mM KCl, 5 mM $MgSO_4$, 5 mM Na-pyruvate, 20 mM glucose, 20 mM taurine, and 10 mM HEPES at pH 6.9.[5] The cultures were incubated in 1 mg/ml collagenase B (Roche Molecular Biochemicals, Indiana Polis, IN) in the low-calcium solution supplemented with 30 μM $CaCl_2$ at 37^{0}C for 1-2 h. Cells were then resuspended in a solution containing 85 mM KCl, 30 mM K_2HPO_4, 5 mM $MgSO_4$, 1 mM EGTA, 2 mM

Na_2ATP, 5 mM Na-pyruvate, 5 mM creatine, 20 mM taurine, and 20 mM glucose at pH 7.2 and incubated at 37^0C for 15 min for more complete dissociation. After dissociation, the cells were plated and cultured in differentiation medium or applied to a Percoll gradient as described below. Cells may also be dissociated with 0.56 units/ml Blendzyme IV (Roche, Indianapolis, IN) at 37^0C for 30 min.

Immunostaining

Immunostaining of EB outgrowth cultures or dissociated cardiomyocytes was performed as follows. Differentiated cultures were fixed in methanol/acetone (3:1) at -20^0C for 20 min for most of the antibodies unless noted below. For staining using antibodies against human Ki-67, cardiac α/β MHC, β-MHC, AFP, or β-tubulin III together with antibodies against cTnI, cells were fixed with freshly prepared 2% paraformaldehyde (PFA) at room temperature (RT) for 20 min followed by permeabilization with 100% ethanol at RT for 5 min. Cells were then washed 2 times with PBS, blocked with 5% normal goat serum (NGS) in PBS at 4^0C overnight, followed by incubation at RT for 2 h with primary antibodies (Table 1 Online) diluted in the primary antibody diluting buffer (Biomeda Corp., Foster city, CA) or 1% NGS in PBS. After washing, the cells were exposed to the corresponding FITC or Texas Red-conjugated secondary antibodies diluted in 1% NGS in PBS at RT for 30-60 min. The cells were then washed again, stained with DAPI and mounted with Vectashield medium (Vector Laboratories Inc. Burlingame, CA) for photomicroscopy.

For double staining with antibodies against sMHC and BrdU, cells in fraction III and IV were replated, cultured for additional 2 days and then pulse-labeled with BrdU for 24 hr. Cells were then fixed with methanol/acetone, blocked, incubated with antibodies against sMHC and secondary antibodies as described above. The cells were then re-permeabilized with 2 N HCl at RT for 10 min, washed 3 times with 5% NGS in PBS, and neutralized with 0.1 M sodium borate at RT for 10 min. After washing with a buffer containing 5% NGS, 1% Triton X-100 and 2 mg/ml BSA in PBS, cells were incubated with antibodies against BrdU diluted in the same buffer at RT for 30 min, followed by washing and secondary antibody incubation. Cells were stained with DAPI and mounted.

Double staining with antibodies against SSEA-4 or Tra 1-81 together with antibodies against cTnI was performed by first staining live cells with SSEA-4 or Tra 1-81 diluted in warm KO DMEM at 37^0C for 30 min. After washing with warm KO DMEM, cells were fixed in 2% PFA at RT for 15 min, washed with PBS and incubated with FITC-conjugated secondary antibodies at RT for 30 min. Cells were washed, re-fixed in 2% PFA, permeabilized with 100% ethanol and then stained with antibodies against cTnI as for other procedures.

To confirm specificity of cTnI staining, individual contracting foci in the differentiated cultures were photographed to record contracting areas before the culture was fixed. The culture was then stained for cTnI immunoreactivity and matched to the original photographs to determine the percentage of contracting areas with cTnI immunoreactivity.

The percentage of sMHC, cTnI, Ki-67 or BrdU immunoreactive cells was determined by counting cells in triplicate wells using 10 images from each well. These values were summed and presented as mean ± standard deviation of cells from 3 wells (300-700 cells were counted per condition).

RT-PCR

Standard real time reverse transcription reactions were performed with a Taqman 7700 Sequence detection system for relative quantification of cardiac α–MHC. Taqman one step RT-PCR master mix (Applied Biosystems, Foster City, CA) was applied using the following reaction conditions: RT at 48^0C for 30 min; denaturation and AmpliTaq gold activation at 95°C for 10 min; amplification for 40 cycles at 95° for 15 sec and 60^0C for 1 min. 18S ribosomal RNA was amplified to serve as a control using a kit for Taqman ribosomal RNA control reagents (Applied Biosystems). Primers for cardiac α-MHC are 5'GGAGGAGCAAGCCAACACCAA3' and 5'GCAGTGAGGTTCCCGTGGCA3'. The probe for cardiac α-MHC is 5'GCGGACATCGCCGAGTCCCAGGTCAA3'. 100 ng-1 pg of 10-fold serially diluted human heart RNA (Clontech) was used to generate a standard curve for the level of cardiac α-MHC. Reactions were analyzed by ABI Prism 7700 Sequence Detection system and the quantitation of expression of cardiac α-MHC was determined by comparison with the standard curve. Similarly, values for 18S were obtained for each sample. Relative α-MHC levels were presented as mean ± standard deviation of the ratio of α-MHC and 18S from triplicate reactions for each sample.

Semiquantitative RT-PCR for Nkx2.5, ANF and GAPDH was performed using standard procedures described elsewhere.[6] Three μg RNA samples were converted into cDNA, serially diluted 1:10 and analyzed for expression of specific genes by PCR analysis.[6] Primers for Nkx2.5 are 5'TGGCTACAGCTGCACTGCCG3' and 5'GGATCCATGCAGCGTGGAC3' Primers for ANF are 5'TAGGGACAGACTGCAAGAGG3' and 5'CGAGGAAGTCACCATCAAACCAC3'. Primers for GAPDH have been described.[7]

***In vitro* responses to pharmacological agents**

EBs were plated onto gelatin or poly-L-lysine-coated 24-well plates and allowed to further differentiate. A day before the experiment, cells were fed with 1 ml/well differentiation medium and beating areas were photographed and marked for later identification. The next day, the beating frequency for each area was measured by visual inspection in a 37^0C microscope chamber before adding the drugs. To examine pharmacological responses, drugs (all from Sigma) were added to the culture at the lowest dose. Cultures were then incubated at 37^0C in the incubator without shaking for 20-30 min. Each culture was then placed in a 37^0C microscope chamber and the beating frequency was monitored. The procedure was repeated several times by sequentially adding additional doses of the drug followed by monitoring the beating frequency. The results are presented as the mean pulsation rate $\pm$ standard error of the mean measured for

10-20 beating areas. Significance was assessed by ANOVA test using StatView: * p< .05, ** p<.005, *** p<.0005.

Results

Effect of DMSO and RA on cardiomyocyte differentiation

In order to enhance cardiomyocyte differentiation, the effect of differentiation induction reagents was evaluated. DMSO has been shown to induce cardiomyocyte differentiation in mEC P19 cells,[8] although it is not required for cardiac differentiation of mES cells. The effect of DMSO on hES cell differentiation was examined by treating EBs from differentiation day 0 to 4 with 0.5-1.5% DMSO. Fewer beating areas were found in cultures treated with 0.5% DMSO compared with non-treated cultures. No beating cells were observed in cultures treated with 0.8%, 1% or 1.5% DMSO. Furthermore, DMSO was toxic to the cells at 1.5%. In addition, real time RT-PCR analysis showed that the level of cardiac α-MHC in 0.5 or 0.8% DMSO treated cells was 4 times lower compared to control untreated cultures. Thus, DMSO treatment decreased cardiomyocyte differentiation from hES cells. We also evaluated retinoic acid (RA), a reagent that enhances cardiogenesis of mES cells[9] and expression of cardiac α-actin in hES cells.[10] RA was added to hES cell cultures at different doses and different times of differentiation (day 0 to 4, 4 to 8, 8 to15 and 4 to15). Treatment with 10^{-9} –10^{-5} M RA at day 0 to 4 was toxic to the cells and did not improve cardiomyocyte differentiation when added to the culture at later times of differentiation. Therefore, DMSO and RA did not enhance H1 hES cell

cardiomyocyte differentiation, in contrast to the positive effect these compounds have on mEC and mES cardiomyocyte differentiation, respectively.

Effect of 5-aza-dC on cardiomyocyte differentiation

As stated in the text, 5-aza-dC treatment at differentiation day 6 to 8 increased levels of α-MHC. To determine whether the increase in α-MHC correlates with an increase in the number of cardiomyocytes, we performed the following experiments. H7 cells were induced to differentiate, treated with 10 μM 5-aza-dC at differentiation day 6 to 8, dissociated at differentiation day 13, replated, cultured for additional 2 days and assessed. We found that the 5-aza-dC treated culture contained 44 ± 2% of cTnI-positive cells while the control culture contained 15 ± 4% cTnI-positive cells. A second experiment using H7 cells at differentiation day 20 showed that the percentage of cTnI-positive cells was 26 ± 2% in 5-aza-dC treated culture and 11 ± 3% in the control culture.

References:

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998;282:1145-1147.
2. Amit M, Carpenter MK, Inokuma MS, Chiu CP, Harris CP, Waknitz MA, Itskovitz-Eldor J, Thomson JA. Clonally derived human embryonic stem cell lines maintain pluripotency and proliferative potential for prolonged periods of culture. *Dev Biol*. 2000;227:271-8.
3. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. Feeder-free growth of undifferentiated human embryonic stem cells. *Nature Biotech*. 2001;19:971-974.
4. Robertson EJ. *Teratocarcinomas and Embryonic Stem cells: A Practical Approach*. Washington D.C.: IRL Press; 1987.
5. Maltsev VA, Rohwedel J, Hescheler J, Wobus AM. Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. *Mech Dev*. 1993;44:41-50.

6. Xu C, Liguori G, Adamson ED, Persico MG. Specific arrest of cardiogenesis in cultured embryonic stem cells lacking Cripto-1. *Dev Biol.* 1998;196:237-47.
7. Hummler E, Barker P, Gatzy J, Beermann F, Verdumo C, Schmidt A, Boucher R, Rossier BC. Early death due to defective neonatal lung liquid clearance in alpha-ENaC-deficient mice. *Nat Genet.* 1996;12:325-8.
8. Edwards MK, Harris JF, McBurney MW. Induced muscle differentiation in an embryonal carcinoma cell line. *Mol Cell Biol.* 1983;3:2280-6.
9. Wobus AM, Kaomei G, Shan J, Wellner MC, Rohwedel J, Ji G, Fleischmann B, Katus HA, Hescheler J, Franz WM. Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. *J Mol Cell Cardiol.* 1997;29:1525-39.
10. Schuldiner M, Yanuka O, Itskovitz-Eldor J, Melton DA, Benvenisty N. Effects of eight growth factors on the differentiation of cells derived from human embryonic stem cells. *Proc. natl. Acad. Sci. USA.* 2000;92:11307-11312.

Table 1 Online. Primary antibodies for cardiomyocyte analysis

Antibodies	Source	Species	Isotypes	Dilutions
cTnI	Spectral Diagnostics	mouse	IgG_1	1:400-500
tropomyosin	Sigma	mouse	IgG_1	1:100
cTnT	Sigma	mouse	IgG_1	1:200
β_1-AR	Santa Cruz Biotech	rabbit	IgG	1:800
α-actinin	Sigma	mouse	IgG_1	1:200
N-cadherin	R&D	rabbit	IgG	1:800
sMHC	Hybridoma Bank	mouse	IgG_{2b}	1:500
α/β MHC	Chemicon	mouse	IgG_{2a}	1:10
β MHC	Chemicon	mouse	IgG_{2a}	1:10
desmin	NeoMarkers	mouse	IgG_1	1:200
GATA-4	Santa Cruz Biotech	rabbit	IgG	1:100
MEF-2A	Santa Cruz Biotech	rabbit	IgG	1:100
myoglobin	Santa Cruz Biotech	goat	IgG	1:500
AFP	Sigma	mouse	IgG_{2a}	1:500
β-tubulin III	Sigma	mouse	IgG_{2b}	1:1000
SSEA4	Hybridoma Bank	mouse	IgG_3	1:5
Tra 1-81	Dr. Peter Andrews	mouse	IgM	1:20
CK-MB	Spectral Diagnostics	mouse	IgG_1	1:100
BrdU	Caltag Lab	mouse	IgG_1	1:100
Ki-67	Dako	rabbit	IgG	1:100

Note: Antibodies against MEF-2A react with members of MEF-2 family. Antibodies against GATA4 and β1-AR are generated using specific peptides for these antigens based on information from Santa Cruz. We thank Dr. Peter Andrews for the Tra 1-81 antibodies.

Table 2 Online. Separation of hES cell-derived cardiomyocytes by Percoll gradient

Fraction	Cells collected	Beating cells*	% sMHC-positive cells	
			Day 2	Day 7
Input cells	1-2 x 10^7	+	17 ± 4%	15 ± 4%
I	9 x 10^6	±	3 ± 1%	3 ± 3%
II	1.6 x 10^6	±	5 ± 1%	2 ± 1%
III	4 x 10^6	++	36 ± 3%	28 ± 9%
IV	1.3 x 10^6	+++	70 ± 5%	52 ± 15%

H7 cell(passage 26)-derived cardiomyocytes differentiated for 21 days were enriched by Percoll gradient separation (see methods). After separation, each layer was collected, and cells were counted and replated. Cultures were maintained for 2 or 7 days before evaluation of sMHC immunoreactivity. Amount of beating cells: +++ > ++ > + >±.

Table 3 Online. Characterization of Percoll-enriched cells

Markers	Cardiac cells	Non-cardiac cells	Cells/Passage#	Days of differentiation
cTnI	++	-	H1 p30	19 + 6
			H7 p45	26 + 2, 26 + 10
			H7 p47	12 + 2, 12 + 10
			H7 p37	13 + 2, 13 + 5
			H7 p34	20 + 7
α/βMHC	++	-	H7 p47	12 + 2
βMHC	++	-	H7 p45	26 + 2
sMHC	++	-	H1 p30	19 + 6
			H7 p47	12 + 10
			H7 p37	13 + 2
			H7 p37	29 +4
N-cadherin	++	±	H7 p47	12 + 10
Myogenin	-	-	H7 p47	12 + 10
SMA	++	+	H7 p37	13 + 2
AFP	-	-	H7 p47	12 + 2
β-tubulin III	-	-	H7 p45	26 + 2
Ki-67	+	+	H7 p47	12 +10, 12 + 2
			H7 p45	26 + 2, 26 + 10
			H7 p37	29 + 4
BrdU	+	+	H7 p37	13 + 2
			H7 p37	29 +4
SSEA4	-	-	H7 p37	13 + 5
Tra1-81	-	-	H7 p34	20 + 7

Summary of multiple experiments in which hES cell-derived cardiomyocytes were enriched using Percoll gradient separation. Cells from fraction III and IV were harvested and evaluated immunocytochemically at the time points indicated. For Myogenin or BrdU staining, sMHC-positive cells were considered as cardiac cells. Ki-67 positive cells were examined in sMHC or cTnI stained cells. For all other markers, cardiac cells were identified as cTnI-positive cells. ++: positive signal detected in all cardiac cells examined. +: positive signal detected in a subset of the cells. ±: the majority of the cells were negative but few showed positive signal. -: no signal detected. Days of differentiation are indicated as x + y: cells were dissociated and separated by Percoll at differentiation day x, plated on laminin-coated chamber slides and cultured for an additional y days.